Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters xx (2006) 1–10
CANCER Letters
www.elsevier.com/locate/canlet

# Real-time telomerase assay of less-invasively collected esophageal cell samples

Brenna M. McGruder [a], Donald H. Atha [a], Wendy Wang [b], Konrad Huppi [c], Wen-Qiang Wei [d], Christian C. Abnet [e], You-Lin Qiao [d], Sanford M. Dawsey [e], Philip R. Taylor [e], John P. Jakupciak [a,*]

[a] *Biochemical Science Division, National Institute of Standards and Technology, 100 Bureau Drive, MS 8311, 20899, Gaithersburg, MD, USA*
[b] *Cancer Biomarkers Research Group, National Cancer Institute, Rockville, MD, USA*
[c] *Advanced Technology Center, National Cancer Institute, Gaithersburg, MD, USA*
[d] *Department of Cancer Epidemiology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China*
[e] *Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MA, USA*

Received 30 August 2005; accepted 4 December 2005

**Abstract**

Genomic and proteomic efforts have discovered a complex list of biomarkers that identify human disease, stratify risk of disease within populations, and monitor drug or therapy responses for treatment. Attention is needed to characterize these biomarkers and to develop high-throughput technologies to evaluate their accuracy and precision. Telomerase activity is correlated with tumor progression, indicating cells that express telomerase possess aggressive clinical behavior and that telomerase activity could be a clinically important cancer biomarker. Traditionally, the detection of cancer has involved invasive procedures to procure samples. There is a need for less invasive approaches suitable for population- and clinic-based assays for cancer early detection. Esophageal balloon cytology (EBC) is a low-invasive screening technique, which samples superficial epithelial cells from the esophagus. Since telomerase activity is absent in superficial cells of normal esophageal squamous epithelium but is often present in superficial cells from dysplastic lesions and ESCCs, measuring telomerase activity in EBC samples may be a good way to screen for these lesions. The development of rapid real-time telomerase activity assays raises the possibility of extending such screening to high-risk populations. In this study, we evaluate the feasibility of using rapid Real-Time Telomerase Repeat Amplification Protocol (RTTRAP) for the analysis of NIST telomerase candidate reference material and esophageal clinical samples. The telomerase activity of eight EBC samples was also measured by capillary electrophoresis of RTTRAP products, RApidTRAP, and hTERT mRNA RT-PCR assays. These findings demonstrate the feasibility of using the RTTRAP assay in EBC samples and suggest that individuals from high-risk populations can be screened for telomerase activity.


*Keywords:* Real-time TRAP; Telomerase activity; Diagnostics; Biomarkers; Telomerase candidate reference material; Esophageal squamous cell carcinoma; Esophageal balloon cytology

## 1. Introduction

The development of biomarkers, their validation and translation to clinical application is becoming increasingly important for the diagnostic community [1].

* Corresponding author. Tel.: +1 301 975 4098; fax: +1 301 975 8505.
*E-mail address:* johnj@nist.gov (J.P. Jakupciak).

0304-3835/$ - see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2005.12.012

The search for cancer-associated molecules has led to the discovery of many molecular markers associated with clinical outcome [2,3]. Among more than 100 validated cancer biomarkers, telomerase is unique [4]. Telomerase activity is the single most common cancer biomarker, detected in 80–90% of cancers [5–15]. In some tissues, telomerase activity was detected at the preneoplastic or in situ stage, suggesting that telomerase activity measurements have potential for early detection applications and telomerase is increasingly being considered as a target for cancer prevention and therapeutics [16]. In other cases, telomerase activity increased with cancer progression, suggesting that telomerase activity measurements could function as a prognostic indicator of patient outcome.

Esophageal cancer is the sixth leading cause of cancer death worldwide [17]. Most cases occur in developing countries, are squamous cell carcinomas and are diagnosed at an advanced stage when therapy with curative intent is not possible. There is great interest in the development of a primary screening test to identify patients with premalignant or early malignant disease, which could be more readily cured. Studies from our group have demonstrated that squamous dysplasia is the precursor lesion for esophageal squamous cell carcinoma (ESCC) [18] and that these lesions can be observed during endoscopy with Lugol's iodine staining [19], but endoscopy is too invasive and too expensive to serve as a primary screening exam even in very high risk populations. Previous efforts to develop a less invasive and more cost-effective primary screening test for ESCC have focused on EBC, a process analogous to a pap smear, but to date this technique has had insufficient sensitivity for detecting squamous dysplasia and cancer [20]. It is evident that molecular high-throughput approaches are needed to develop a more sensitive, less invasive and objective test for mass screening of high-risk populations. Telomerase activity and hTERT mRNA appear to be absent in the outer epithelial layers of normal esophageal squamous epithelium, but they increase in preneoplastic lesions and squamous cell carcinomas [21,22], so they may be useful markers in a molecular screening test for these lesions and they may improve the clinical utility of EBC exams. Advances in molecular biology have provided a greater understanding of cancer progression including the use of biomarkers of early detection and risk assessment [23]. Automated and high-throughput assays and reference materials have recently advanced the utility of telomerase as a diagnostic for such early detection [24,25].

Early detection of disease is a major focus by which to improve outcomes for patients with solid tumors [3,26,27]. Further, biomarker discovery and validation is in great demand by the pharmaceutical and healthcare industries. High-throughput [28] and global scanning [29] methods that are low-cost and reliable clinical platforms are essential to analyze large cohort studies, which can statistically validate potential DNA, RNA and protein biomarkers for early detection assays. Standardized controls or reference materials will be essential for the acceptance of data collected from these new technology platforms by regulatory agencies.

In partnership with the National Cancer Institute's Early Detection Research Network (EDRN), the National Institute of Standards and Technology (NIST) evaluates analytical methods for the validation of biomarkers associated with the detection of cancer and optimizes high-throughput technologies for clinical applications [28–31]. The mission is to validate biomarkers for reliable results and demonstrate proof-of-principle of their translation into clinical applications. Then, private sector or other government agencies can promote the use of the biomarkers and clinically relevant technologies in clinical trials and can incorporate NIST standards and guidelines to establish a diagnostic.

The combination of less-invasive collection techniques such as EBC and rapid diagnostic assays are central components for population screening [32]. It is critical that populations at-risk for cancer have a real-time based assay. Although automated TRAP assays have been developed, many difficulties with TRAP assays have prevented their wide acceptance in clinical settings. Several drawbacks include: TRAP assays are multi-step, time-consuming, and require extensive post-PCR processing to determine if the potential cancer sample has signal greater than the negative control and activity comparable to the positive control. These problems have been resolved with real-time TRAP assays.

Recently, we reported the development of a fluorescent-based, real-time assay to measure telomerase activity [24]. This one-step procedure simplifies the TRAP assay. RTTRAP is less cumbersome, maintains high-throughput, and obviates the need for extensive post-PCR processing. Further, RTTRAP compressed the assay time to 1 h as compared to at least 10 h with traditional protocols [24,33]. In the current study we measured the telomerase activity of eight EBC specimens from a population at high risk for esophageal cancer and we compared these activity measurements to the number of hTERT mRNA transcripts and to other

TRAP methods. Our findings suggest potential for RTTRAP techniques in telomerase-based primary screening assays using similar samples.

## 2. Materials and methods*

*Certain commercial equipment, instruments, materials, or companies are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation nor endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are the best available for the purpose.

### 2.1. Esophageal balloon cytology samples

Balloon cytology sampling of the esophagus was performed in Linxian, China, as previously described [20]. The sampling was approved by the Institutional Review Boards of the Cancer Institute of the Chinese Academy of Medical Sciences and the US National Cancer Institute, and each patient signed an informed consent before the procedure. Before the examination, the patients were given a 2% lidocaine slurry by mouth for local anesthesia. The deflated mesh-covered balloon was then passed through the esophagus into the stomach, where it was inflated with 20–30 ml of air and then pulled back up the esophagus. At 18 cm from the incisor teeth, the balloon was deflated and withdrawn. After removal, the balloon was placed in 40 ml of saline in a 50 ml centrifuge tube, vortexed to remove adherent cells, and discarded. The cell solution was centrifuged, the supernatant discarded, and the pellet snap-frozen and stored in liquid nitrogen. The frozen pellets were transferred to NCI for further evaluation.

### 2.2. Cell culture for telomerase candidate reference material

Stock cultures of A549 cells (ATCC, Manassas, VA, USA) were grown as previously described using standard laboratory techniques [28,34].

### 2.3. Preparation of total RNA

Total cellular RNA was extracted from A549 cells as previously described using a modified phenol procedure [28,34]. The cells were centrifuged and the resulting pellet was resuspended in 750 μL of TRIzol per $5\times10^6$ cells. The concentration of total RNA was calculated based on $OD_{260}$ measurements as a means to address RNA yield only. The spectrophotometric value for the total RNA was divided by the number of cells used in the extraction to determine the amount of RNA per cell. The coefficient of variation was greater for the O.D. measurement of total RNA (CV = 71%) than for real-time PCR (CV = 27%). The hTERT mRNA amount per cell was taken directly from the real-time PCR results.

### 2.4. Measurement of hTERT mRNA

The RT-PCR assay for hTERT mRNA, the transcript that encodes for the catalytic subunit of the holoenyzme telomerase, has potential for cancer detection from bodily fluids and paraffin embedded archived samples. Because the balloon cytology specimen was processed to obtain DNA, RNA, and cell extracts in parallel, we measured the level of transcript abundance from the same subjects. The number of hTERT mRNA molecules was determined by real-time RT-PCR as previously described using the LightCycler Telo-TAGGG kit from Roche Molecular Biochemicals (Indianapolis, IN) [28]. RNA was converted to cDNA and specific gene primers were used to amplify hTERT mRNA as its full-length product. Melting curve analysis confirmed the amplification of one pure product. Quantification of the product was obtained by extrapolating the data against a standard curve run in triplicate. Human porphobilinogen deaminase (PBGD) mRNA was chosen as the housekeeping gene. RNA molecules from telomerase positive cells (A549) were detected from 100,000 to a lower limit of 10 cells. Dilutions of 1 cell equivalent did not contain a detectable product. The Linxian clinical samples were normalized to 20 ng/ml of total RNA before being measured by RT-PCR.

### 2.5. The TRAP assay system

Amplified products were generated by the TRAP assay as previously described using the following protocol [24,28]. The Linxian clinical samples were normalized to 18 μg/mL of total protein and serially diluted to 1 ng/mL before analysis by TRAP/PCR methods. Clinical samples may contain inhibitors of telomerase activity. These inhibitors can vary depending on the clinical sample. It is therefore difficult to prescribe an optimal dilution, but instead samples should be compared in a wide range of dilution. Hence, serial dilutions are recommended.

#### 2.5.1. Telomerase extension reaction

Two microlitres of cell lysate is added to 23 μL of a solution containing 1× PCR buffer, 200 μmol/L 2′-deoxy-nucleoside 5′-triphosphates (dNTP's, 50 μmol/L each), and 200 ng of the telomerase substrate (TS primer). The solution is incubated at 30 °C for 30 min.

#### 2.5.2. PCR amplification step

To the extension reaction is added 25 μL of a solution containing 1× PCR buffer, 200 μmol/L dNTPs, 3.75 units Taq polymerase, 100 ng of the reverse CX primer. PCR for the TRAP assay was carried out in a Perkin Elmer 9600 Thermal Cycler using the following program: 95 °C for 15 min; 36 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min; 4 °C hold.

*Primer sequences*

CX primer: 5′-CCCTTACCCTTACCCTTACCCTAA-3′.

*TS primer*

5′-AATCCGTCGAGCAGAGTT-3′. Fluorescently-labeled TRAP PCR products were produced using equivalent amounts of HEX (4,7,2′,4′,5′,7′,-hexachloro-6-carboxyfluorescein), 5′ end-labeled TS and unlabeled CX primers (PE/Applied Biosystems).

The TRAP/PCR was automated as previously described using the MWG RoboSeq SE 4204 [24]. This 'RApidTRAP' system allowed a more efficient method for consistent high-throughput measurements [28]. The program used solution volumes sufficient for multiple determinations in the 96-well plate format.

### 2.6. Real-time TRAP assay

#### 2.6.1. TRAP extension reaction

One microlitre of cell lysate, containing a specified number of cells or total protein concentration, was added to 23 μl of a solution containing 1× PCR buffer (Qiagen, Valencia, CA), 200 μM dNTP's (50 μM each), and 200 ng of the telomerase substrate (TS primer). The solution was incubated at 30 °C for 30 min.

#### 2.6.2. Real-time PCR

Real-time PCR was performed after the first step of the TRAP extension reaction. To the extension reaction is added 10.9 μl of solution containing 10 μl of IQ Supermix (BioRad, Hercules, CA), 0.1 μl (100 ng) of the reverse CX primer and 0.8 μl of (30 uM)Taqman FAM/TAM-labeled probe. Fluorescence was detected in real-time using the Bio-RAD iCycler iQ Real time PCR Detection System (Bio-Rad) using the following program: 95 °C for 2.5 min; 50 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 4 °C hold.

#### 2.6.3. Primer and probe sequences

TS: 5′-AATCCGTCGAGCAGAGTT-3′;
CX: 5′-CCCTTACCCTTACCCTTACCCTTAA-3′;
TaqMan (probe): FAM 5′-CCCTTACCCTTACCCTTA-3′ TAM.

### 2.7. Capillary electrophoresis measurements

Fluorescently-labeled RTTRAP and RApidTRAP PCR products were analyzed by capillary electrophoresis as previously described [28] by combining 1 μL of PCR product with 10.5 μL deionized formamide, and 0.5 μL of ROX-500—labeled internal size standard (PE/Applied Biosystems). Separations were performed using the PE/Applied Biosystems Model 3100 multicapillary instrument using the PE/Applied Biosystems GeneScan capillary array (50 cm × 50 μm) and the POP4 polymer system as described previously [24,28]. Samples were electrokinetically injected (10 s, 15 kV) and separated at 5.0 kV at a temperature of 60 °C. The amount of extension product (incorporated primer) was calculated for each sample as the total summed area of labeled peaks corresponding to the extension products.

## 3. Results

In this study, we evaluated the telomerase activity of cell extracts recovered from balloon cytology as a potential less-invasive diagnostic tool. The telomerase activity of each cell extract was measured by RTTRAP and also measured across several different TRAP technologies and platforms. TRAP methods, RApidTRAP and hTERT mRNA measurements, independently confirmed the results obtained from RTTRAP. Table 1 shows the characterization of the clinical samples using Real-Time detection, capillary electrophoresis (CE) of the RTTRAP products (telomeric repeats), and hTERT RT-PCR mRNA quantification. The standard deviation for the RTTRAP measurements ranged from 0.4 to 4 (CV% 4–16%). The standard deviation for the CE measurements ranged from 5 to 46 (CV% 11–21%). The standard deviation for the hTERT

Table 1
Comparison of telomerase activity, CE peak area, and hTERT mRNA measurements

| Sample | Telomerase (RTTRAP) Ct | SD | CE of RTTRAP products peak area | SD | Telomerase hTERT mRNA copy number | SD |
|---|---|---|---|---|---|---|
| 738 | 12.2 | 1.4 | 100 | 14 | 144 | 15 |
| 740 | 10.9 | 1.0 | 166 | 28 | 97 | 11 |
| 082 | 9.7 | 0.4 | 407 | 46 | 762 | 37 |
| 130 | 12.5 | 1.4 | 121 | 16 | 163 | 15 |
| 212 | 15.3 | 2.0 | 23 | 5 | 61 | 9 |
| 346 | 14.4 | 1.5 | 23 | 5 | 58 | 11 |
| 893 | 11.8 | 1.1 | 162 | 22 | 246 | 26 |
| 419 | 21.1 | 4.0 | 23 | 5 | 22 | 6 |
| Cancer cRM | 10.7 | 0.5 | 175 | 18 | 490 | 14 |

Table 2
Telomerase activity of A549 cell lysate measured by RApidTRAP

| Concentration cell equivalents | Activity total peak area |
|---|---|
| Blank | 24.05 |
| 1 | 26.23 |
| 10 | 27.87 |
| 100 | 34.88 |
| 1000 | 64.83 |
| 2000 | 101.0 |
| 10,000 | 119.9 |

mRNA measurements ranged from 6 to 37 (CV% 1.7–27%). A standard curve of telomerase activity using the RApidTRAP system and the A549 candidate reference material is shown in Table 2. As previously shown, the activity (total peak area) is linear in the range of about 1–1000 cell equivalents.

Fig. 1 shows results of real-time TRAP analysis of telomerase activity contained in eight EBC cell samples. The average Ct values are plotted with their standard deviations. Sample 82, with high telomerase activity, was detected early in the assay, whereas samples 212 and 419, with little or no telomerase activity, were detected later. The no-template control did not display a signal even after 50 cycles, as expected.

The results of RTTRAP were confirmed by capillary electrophoretic analysis of the RTTRAP synthesized telomeric amplicons (Fig. 2). The expected TRAP ladder of extension products ranged from 40 to 150 bp at characteristic 6 bp intervals. In addition, the measured hTERT mRNA concentration indicates a correlation with abundant hTERT mRNA and telomerase activity. Fig. 3 shows the results of the hTERT mRNA analysis of the same samples analyzed by RTTRAP and multi-capillary electrophoresis. When the activity and the hTERT copy number are compared, samples with high concentrations of hTERT mRNA, sample 82, possessed strong telomerase activity and samples that had low hTERT mRNA copies, sample 419, possessed weak telomerase activity. Overall, the four different platforms ranked the samples quite similarly by telomerase activity and mRNA copy number (Table 3).

## 4. Discussion

In this study, we evaluated telomerase activity and hTERT mRNA in samples obtained by EBC in a population at high risk for ESCC. This was an initial step in evaluating the feasibility of screening for esophageal precursor lesions and early ESCC based on telomerase activity. The telomerase activity of each sample was measured in parallel by a new real-time TRAP assay (RTTRAP) and by other telomerase technologies and platforms. The RApidTRAP and hTERT mRNA measurements were concordant with the results obtained by RTTRAP.

The clinical community and patient advocacy groups have been asserting the need for rapid analysis of potentially cancerous tissues obtained using less-invasive procedures. Over the last few years, the role of biomarker analysis has been changing from that of an ancillary diagnostic technique to that of a stand-alone diagnostic method [35]. There is also growing sentiment that there already are effective biomarkers, but that they have only been exhaustively evaluated for use on end-stage cancer and not for early detection. Prominent among biomarkers is the use of telomerase activity as a potential diagnostic for cancer. Although TRAP assays and subsequent improvements have been validated as accurate technologies for the detection of telomerase activity, all remain cumbersome and require

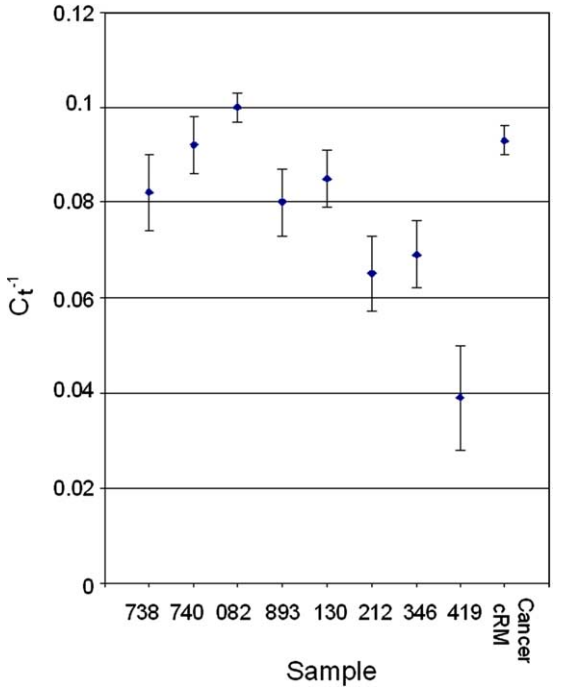


Fig. 1. Real-time inverse plot of RTTRAP Ct results. The average Ct scores for the balloon cytology samples and the telomerase candidate reference material are plotted with their standard deviations. Because real-time assays display samples with a higher concentration of analyte as a smaller Ct, inverse values are given to illustrate the data in the same orientation as is shown in Fig. 4. Points closer to the top of the plot indicate strong telomerase activity, whereas lower results indicate weak or no telomerase activity. cRM represents the telomerase candidate reference material.

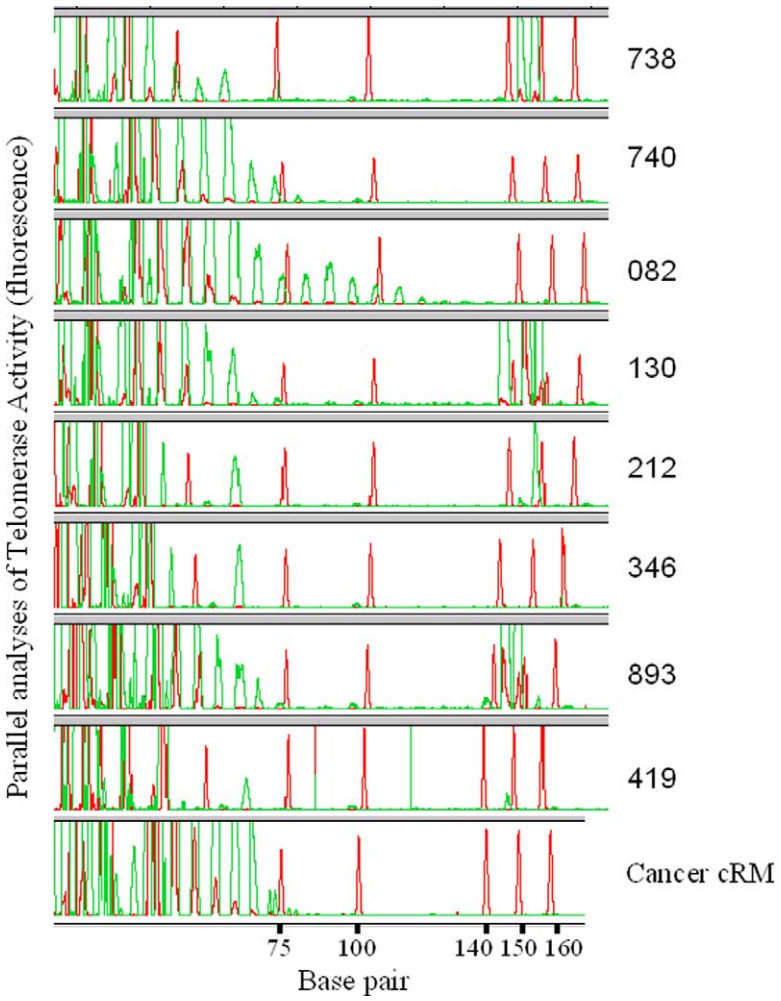


Fig. 2. Multi-capillary electrophoretic analysis of RTTRAP telomerase-generated amplicons ($T_2AG_3$ extension products). The processivity and relative amount of telomerase was compared for each sample. The assay was performed using fluorescent labeled primers as described previously [24]. The green peaks represent the $T_2AG_3$ aggregates generated by the inherent telomerase concentration present in the sample. The extension products ranged from 40 to 150 in 6 bp uniform increments. Red peaks represent DNA size markers (ROX 500), with the exception of some crossover pullup below 75 bp. Electropherograms were produced for separate RTTRAP reactions using the ABI 3100 multi-capillary instrument. Samples that contained extension products larger than 40 bp in size were considered to be telomerase positive. Samples 738, 740, 82, 130, 893, and the cRM have a uniform ladder of TRAP/PCR products. Products 40 bps and smaller are due to primer–dimer adducts between the TS and CS primers, as expected.

rather complex post-analytical data processing prior to interpretation of sample results. The traditional TRAP assay was automated to decrease sample analysis time, address reproducibility and facilitate statistical analysis of large numbers of cohort samples to determine the association of cancer and telomerase activity [28]. To simplify the cumbersome post-PCR processing of RApidTRAP, we [24] and others [36–39]

Table 3
Sample ranking across methods of detection

| Method | Rank 1 | Rank 2 | Rank 3 | Rank 4 | Rank 5 | Rank 6 | Rank 7 | Rank 8 |
|---|---|---|---|---|---|---|---|---|
| RTTRAP | 82 | 740 | 893 | 738 | 130 | 346 | 212 | 419 |
| CE of RTTRAP products | 82 | 740 | 893 | 130 | 738 | 346 | 212 | 419 |
| RApidTRAP | 82 | 740 | 130 | 893 | 212 | 346 | 738 | 419 |
| hTERT | 82 | 893 | 130 | 738 | 740 | 212 | 346 | 419 |

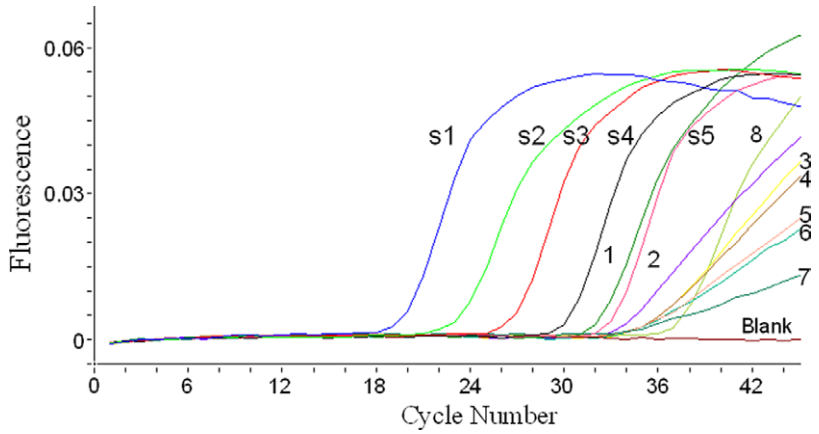


Fig. 3. RT-PCR of hTERT mRNA. The *y*-axis represents the relative ratio of fluorescence between the reference channel and the sample channel. Curves s1–s5 represent the concentration of hTERT transcript detected from the RNA standards. Commercial standards were used to quantify the number of hTERT mRNA molecules (representing $1.28\times10^6$, $1.05\times10^5$, $1.07\times10^4$, 1260, and 190 molecules, respectively). For clarity, sample triplicates are not shown. Increasing to decreasing number of hTERT molecules were observed; 082 (762 hTERT/uL), 893 (246 copies/uL), 130 (163 copies/uL), 738 (144 copies/uL), 740 (97 copies/uL), 212, (61 copies/uL), 346, (58 copies/uL), 419 (22 copies/uL). Curves 1–8, represent samples 82, 893, 346, 740, 212, 738, 130, and 419, respectively.

have developed real-time TRAP assays. Further, a telomerase candidate RM has been developed to normalize telomerase activity measurements [24].

The RTTRAP method is based on the traditional TRAP/PCR reaction and incorporates the same primer strategy. The issues with respect to template quality, probe design, and reaction optimization still apply. Detection of the TRAP/PCR products in real-time can be accomplished by the use of labeled primers or DNA dyes. Unlike other real-time TRAP variations, RTTRAP uses a hydrolysis styled probe, FAM-5′-CCCTTACCCTTACCCTTA-3′-TAM. After duel labeled probe hybridization and degradation by the exonuclease activity of the polymerase, the reporter fluorescence increases proportional to the amount of template. Other real time TRAP variations have used intercalating agents (SYBR Green) or molecular beacon styled probes [33,37]. Real-time variations of the TRAP assay provide a numerical output, high-throughput, and when combined with automated reagent handling, provide rapid analysis. The real-time adaptation of TRAP incorporates specific fluorescent labeled probes in the PCR reaction, which bind to the telomerase synthesized telomeric repeats. The number of telomeric repeats (and hence, the amount of telomerase activity present in the sample) is determined by the fluorescence produced during the extension step of each cycle. The use of different probes, Taq-Man (hydrolysis) or molecular beacons (hairpin), or intercalation of fluorescent dyes, demonstrate that real-time TRAP assays are robust tools to quantify telomerase.

To extend the collaborative-interagency effort between NIST, EDRN and principal investigators at the NCI, four key aspects of cancer diagnostics were united into a pilot study: (1) Use of a most common cancer biomarker (telomerase); (2) Rapid sample analysis (real-time TRAP); (3) Specimens isolated from a population at risk for a common cancer; and (4) Less-invasive sample collection. The results encourage us to pursue real-time measurements of telomerase activity as a potential primary screening assay for early ESCC and its precursor lesions.

Increased expression of telomerase occurs in dysplastic esophageal tissue prior to tumor invasion [40,41], which suggests that increased telomerase activity and/or hTERT expression may be good molecular markers to use in primary screening studies and early detection. In this study, we evaluated RTTRAP with a candidate cancer RM and demonstrated that individuals from an at-risk population can be screened for telomerase activity by a simplified, high-throughput RTTRAP assay. Although our pilot study is small, we identified samples with telomerase activity both greater than and less than our candidate cancer reference material. The reference material will be further characterized for use as a NIST Standard Reference Material via an interlaboratory cross-validation.

The candidate reference material had strong telomerase activity, as expected [28]. This was supported by the Ct score (Fig. 1), the number of $T_2AG_3$ extension products (Fig. 2) and the abundance of hTERT mRNA (Fig. 3). Sample 82 possessed equivalent telomerase

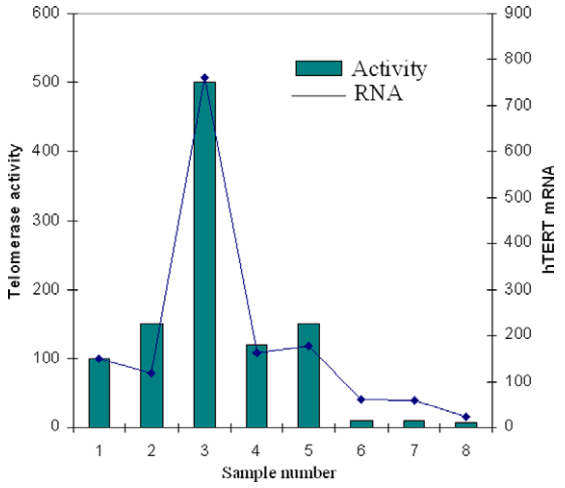


Fig. 4. Comparison of telomerase activity and hTERT mRNA measurements. There is a correlation between telomerase activity (solid bars) and the amount of cellular hTERT mRNA (textured bar). The copy number of hTERT was taken directly from RT-PCR measurements. The telomerase activity (total peak area) was obtained from automated CE measurements. The ratio is expected to vary between different tissue types. Sample numbers 1–8 are samples 738, 740, 82, 130, 893, 212, 346, and 419, respectively.

activity and hTERT mRNA copy number. The TRAP measurements indicate that samples 212, 346, and 419 have no detectable telomeric repeats and are telomerase negative samples (Fig. 2). Although, samples 212, 346, and 419 have a detectable level of hTERT mRNA, it apparently is not sufficient to establish a robust telomerase activity (Figs. 2 and 3, respectively). The samples were ranked according to the degree of telomerase detected by the different detection methods (Table 3). Variations in the sample ranking, as observed in Row 4 (hTERT), which ranks the samples according to the number of mRNA transcripts, in general correlates well with increasing amounts of telomerase activity. However, the relationship between mRNA copy number and telomerase activity is dependent on the translation to the active telomerase protein. Hence, a difference in the ranking result is not surprising. Variations in the ranking results of RApidTRAP, also follow the trend of increasing telomerase activity. However, because RApidTRAP is a multistep assay and involves calculating the areas under the curves, the percent error is greater. This is not surprising and error rates as great as 20%, which may explain the differences of samples measured across platforms, have previously been reported [28]. The most consistent values obtained are the results of RTTRAP and the analyses of the real-time PCR products by capillary electrophoresis. A direct and quantitative determination of telomerase protein (anti-telomerase antibody or mass spectrometry) rather than a total protein assay would help to confirm this. A larger study is currently being designed to evaluate whether the RTTRAP assay can separate individuals with biopsy-proven squamous dysplasia or ESCC from those without this disease and to define an optimal threshold for making this distinction.

To illustrate the relationship between the telomerase activity and hTERT message abundance, we plotted both analyses in Fig. 4. This is particularly important in evaluating whether changes observed in telomerase activity are due to physical changes in the telomerase molecule or because of changes in the mRNA expression. The ratio of telomerase activity to hTERT abundance is expected to vary between different tissue types. This ratio is a measure of the status of telomerase in the cell and at low telomerase levels, helps to confirm its presence. This is consistent with previous RApid-TRAP analyses [28] where a correlation was observed between hTERT mRNA and telomerase activity.

Our data demonstrates that cancer associated biomarkers can be measured in about 1 h (20 min RTPCR, 20 min data analysis, etc.) in samples from individuals of at-risk populations, representing a significant time savings. Moreover, a positive signal can be detected in a few minutes, because, as with all real-time assays, it is not necessary to wait for the entire reaction to reach completion (50 PCR cycles). In the future we intend to define an operating characteristic curve to demarcate the proper threshold that categorizes individuals with and without disease.

RTTRAP has promise to function as a primary assay to triage individuals into at-risk groups and may prove useful for risk stratification and surveillance strategies. Further, RTTRAP is based on a real-time PCR method, which is a clinically accepted technology. Validation studies using other population sets will be necessary to authenticate its use for cancer screening.

## Acknowledgements

This study was a collaborative effort between the National Institute of Standards and Technology (NIST), the National Cancer Institute (NCI), the NCI Early Detection Research Network (EDRN), and the Cancer Institute of the Chinese Academy of Medical Sciences. The study was supported in part by NCI Contract No. N01-RC-91019 and in part by the

Intramural Research Programs of the NCI Division of Cancer Epidemiology and Genetics and the Center for Cancer Research. Clinical samples were received by NIST under a NIST-NCI material transfer agreement. Special thanks go to Dr. Sudhir Srivastava, Director of the EDRN, for helping to coordinate this partnership.

## References

[1] D. Sidransky, Emerging molecular markers of cancer, Nat. Rev. 2 (2002) 210–219.
[2] E. Hiyama, K. Hiyama, Clinical utility of telomerase in cancer, Oncogene 21 (2002) 643–649.
[3] R. Etzioni, N. Urban, S. Ramsey, M. McIntosh, S. Schwartz, B. Reid, et al., The case for early detection, Nat. Rev. 3 (2003) 1–9.
[4] J.E. Joy, E.E. Penhoet, D.B. Petitti (Eds.), Saving women's lives: strategies for improving breast cancer detection and diagnosis, The National Academies Press, Washington, DC, 2005, pp. 155–187.
[5] A.K. Meeker, D.S. Coffey, Telomerase, a promising marker of biological immortality of germ, stem, and cancer cells, Biochemistry 62 (1997) 1323–1331.
[6] N.W. Kim, M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L. Ho, et al., Specific association of human telomerase activity with immortal cells and cancer, Science 266 (1994) 2011–2015.
[7] J.W. Shay, S. Bacchetti, A survey of telomerase activity in human cancer, Eur. J. Cancer 33 (1997) 787–791.
[8] T. Arai, Y. Yasuda, T. Takaya, Y. Ito, K. Hayakawa, S. Toshima, et al., Application of telomerase activity for screening of primary lung cancer in broncho-alveolar lavage fluid, Oncol. Rep. 5 (1998) 405–408.
[9] A. Asai, Y. Kiyozuka, R. Yoshida, T. Fujii, K. Hioki, A. Tsubura, Telomere length, telomerase activity and telomerase RNA expression in human esophageal cancer cells: correlation with cell proliferation, differentiation and chemosensitivity to anticancer drugs, Anticancer Res. 18 (1998) 1465–1472.
[10] M. Engelhardt, P. Drullinsky, J. Guillem, M. Moore, Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer, Clin. Cancer Res. 11 (1997) 1931–1941.
[11] J.S. Dome, S. Chung, T. Bergemann, C.B. Umbricht, M. Saji, L.A. Carey, et al., High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilm' tumor, Cancer Res. 59 (1999) 4301–4307.
[12] Y.I. Zhang, A.S. Zhang, B.P. Wu, D.Y. Zhou, Early diagnosis for colorectal cancer in China, World J. Gastroenterol. 8 (2002) 21–25.
[13] L.Z. Qin, Z.Y. Tang, The prognostic molecular marker in hepatocellular carcinoma, World J. Gastroenterol. 8 (2002) 385–392.
[14] D. Broccoli, J.W. Young, T. de Lange, Telomerase activity in normal and malignant hemotopoietic cells, Proc. Natl Acad. Sci. USA 92 (1995) 9082–9086.
[15] L.L. Smith, H.A. Coller, J.M. Roberts, Telomerase modulates expression of growth-controlling genes and enhances cell proliferation, Nat. Cell Biol. 5 (2003) 474–479.
[16] J.W. Shay, Meeting Report; the role of telomeres and telomerase in cancer, Cancer Res. 65 (2005) 3513–3517.
[17] D.M. Parkin, F.I. Bray, S.S. Devesa, Cancer burden in the year 2000; the global picture, Eur. J. Cancer 37 (Suppl. 8) (2001) S4–S66.
[18] G.Q. Wang, C.C. Abnet, Q. Shen, K.J. Lewin, X.D. Sun, M.J. Roth, et al., Histo Histologic precursors of esophageal squamous cell carcinoma: results from a 13-year prospective follow-up study in a high-risk population, Gut 54 (2005) 187–192.
[19] S.M. Dawsey, D.E. Fleischer, G.Q. Wang, B. Zhou, J.A. Kidwell, N. Lu, et al., Mucosal iodine staining improves endoscopic visualization of squamous dysplasia and squamous cell carcinoma of the esophagus in Linxian, China, Cancer 83 (1998) 220–231.
[20] M.J. Roth, S.F. Liu, S.M. Dawsey, B. Zhou, C. Copeland, G.Q. Wang, et al., Cytologic detection of esophageal squamous cell carcinoma and precursor lesions using balloon and sponge samplers in asymptomatic adults in Linxian, China, Cancer 80 (1997) 2047–2059.
[21] H.P. Yu, S.Q. Xu, W.H. Lu, Y.Y. Li, F. Li, X.L. Wang, Y.H. Su, Telomerase activity and expression of telomerase genes in squamous dysplasia and squamous cell carcinoma of the esophagus, J. Surg. Oncol. 86 (2004) 99–104.
[22] L.F. Zuo, P.Z. Lin, F.Y. Qi, J.W. Guo, J.H. Liu, Flow cytometric analysis of DNA, telomerase content and multi-gene expression in esophageal epithelial dysplasia, World, J. Gastroenterol. 9 (2003) 2409–2412.
[23] C.C. Abnet, K. Huppi, A. Carrera, D. Armistead, K. McKenney, N. Hu, et al., Control region mutations and the common deletion are frequent in the mitochondrial DNA of patients with esophageal squamous cell carcinoma, BMC Cancer 4 (2004) 30–38.
[24] J.P. Jakupciak, P.E. Barker, W. Wang, S. Srivastava, D.H. Atha, Preparation and characterization of candidate reference materials for telomerase assays, Clin. Chem. 51 (2005) 1443–1450.
[25] C.D. O'Connell, J.P. Jakupciak, NIST reference materials for medical testing in: W.B. Coleman, G.J. Tsongalis (Eds.), Encyclopedia of Diagnostic Genomics and Proteomics, Humana Press, New Jersey, 2005, pp. 243–246.
[26] P.D. Wagner, P. Maruvada, S. Srivastava, Molecular diagnostics; a new frontier in cancer prevention, Expert Rev. Mol. Diagn. 4 (2004) 503–511.
[27] M.S. Pepe, R. Etzioni, Z. Feng, J.D. Potter, M.L. Thompson, M. Thornquist, et al., Phases of biomarker development for early detection of cancer, J. Natl Cancer Inst. 93 (2001) 1054–1061.
[28] J.P. Jakupciak, W. Wang, P.E. Barker, S. Srivastava, D.H. Atha, Analytical validation of telomerase activity for cancer early detection: TRAP/PCR-CE and hTERT mRNA quantification assay for high-throughput screening of tumor cells, J. Mol. Diagn. 6 (2004) 157–165.
[29] C.D. O'Connell, L. Tulley, J. Devaney, M.A. Marino, J.P. Jakupciak, D.H. Atha, Renewable standard reference material for the detection of TP53 mutations, Mol. Diagn. 7 (2003) 85–97.
[30] C.D. O'Connell, D.H. Atha, J.P. Jakupciak, J.A. Amos, K.L. Richie, Standardization of PCR amplification for fragile X trinucleotide repeat measurements, Clin. Genet. 61 (2002) 13–20.
[31] S. Srivastava, B.S. Kramer, Early detection cancer research network, Lab. Invest. 80 (2000) 1147–1148.

[32] M. Verma, B.K. Dunn, S. Ross, P. Jain, W. Wang, R. Hayes, A. Umar, Early detection and risk assessment proceedings and recommendations for the workshop on epigenetics in cancer prevention, Ann. NY Acad. Sci. 983 (2003) 298–319.

[33] L.W. Elmore, H.L. Forsythe, A. Ferreira-Gonzalez, C.T. Garrett, G.M. Clark, S.E. Holt, Real-time quantitative analysis of telomerase activity in breast tumor specimens using a highly specific and sensitive fluorescent based assay, Diagn. Mol. Path. 11 (2002) 177–185.

[34] P.E. Barker, in: R.T. Schimke (Ed.), Isolation of Double Minutes from a Human Tumor Cell Line in 'Gene Amplification', Cold Spring Harbor, New York, 1982, pp. 205–212.

[35] T.J. O'Leary, Standardization in immunohistochemistry, Appl. Immunohist. Mol. Morphol. 9 (2001) 1–2.

[36] C. Maesawa, T. Inaba, H. Sato, S. Iijima, K. Ishida, M. Terashima, et al., A rapid biosensor chip assay for measuring of telomerase activity using surface plasmon resonance, Nucl. Acids Res. 31 (2003) e4-4.

[37] M. Hou, D. Xu, M. Bjorkholm, A. Gruber, Real-time quantitative telomeric repeat amplification protocol assay fro the detection of telomerase activity, Clin. Chem. 47 (2001) 519–524.

[38] H. Wege, M.S. Chui, H.T. Le, J.M. Tran, M.A. Zern, SYBR green realtime telomeric repeat amplification protocol for the rapid quantification of telomerase activity, Nucl. Acids Res. 31 (2003) 1–7.

[39] G.I. Botchkina, R.H. Kim, I.L. Botchina, A. Kirshenbaum, Z. Frischer, H.L. Adler, Noninvasive detection of prostate cancer by quantitative analysis of telomerase activity, Clin. Cancer Res. 11 (2005) 3243–3249.

[40] L. Chun, W. Ming-Yao, L. Ying-Rui, W. Xian-Ying, Correlation between expression of human telomerase subunits and telomerase activity in esophageal squamous cell carcinoma, World J. Gastroenterol. 9 (2003) 2395–2399.

[41] E. Hiyama, K. Hiyama, Telomerase as tumor marker, Cancer Lett. 194 (2003) 221–233.